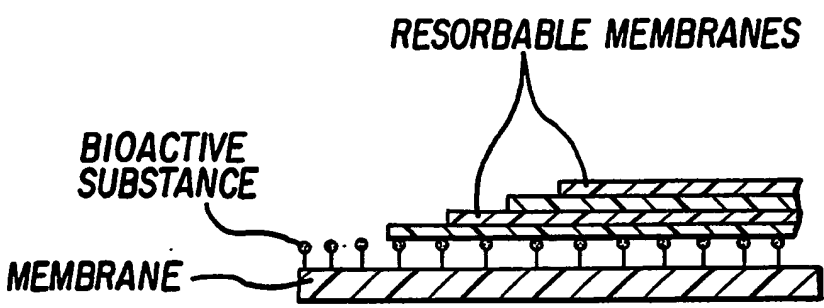


INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 35/32, 37/02, 37/24	A1	(11) International Publication Number: WO 90/13302 (43) International Publication Date: 15 November 1990 (15.11.90)
(21) International Application Number: PCT/US90/02406 (22) International Filing Date: 30 April 1990 (30.04.90) (30) Priority data: 344,632 28 April 1989 (28.04.89) US (60) Parent Application or Grant (63) Related by Continuation US 344,632 (CIP) Filed on 28 April 1989 (28.04.89) (71) Applicant (for all designated States except US): BRIGHAM AND WOMEN'S HOSPITAL [US/US]; 75 Francis Street, Boston, MA 02115 (US).		(72) Inventor; and (75) Inventor/Applicant (for US only) : SONIS, Stephen, T. [US/ US]; 8 Decatur Lane, Wayland, MA 01778 (US). (74) Agents: GOLDSTEIN, Jorge, A. et al.; Saidman, Sterne, Kessler & Goldstein, 1225 Connecticut Avenue, N.W., Suite 300, Washington, DC 20036 (US). (81) Designated States: AT (European patent), AU, BE (Euro- + pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (Eu- ropean patent), SE (European patent), US. Published <i>With international search report.</i>
(54) Title: NOVEL MATERIALS AND METHODS FOR GUIDED TISSUE REGENERATION <div style="text-align: center;">  </div> (57) Abstract <p>Novel materials and methods for guided tissue generation are disclosed. The compositions of the invention comprise a biodegradable membrane comprising a first and a second side, wherein said first side of said membrane comprises one or more biologically active substances, not present on or in said second side. Alternatively, both sides of the membrane may comprise one or more biologically active substances. One or both sides of said membrane may be at least partially coated with a second resorbable membrane layer of varying thickness and/or composition, thereby allowing controlled release of said biologically active substance(s). The compositions, materials and methods of the invention have therapeutic utility for guiding tissue regeneration in animals, including humans.</p>		

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MC	Monaco
AU	Australia	FI	Finland	MG	Madagascar
BB	Barbados	FR	France	ML	Mali
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Fasso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GR	Greece	NL	Netherlands
BJ	Benin	HU	Hungary	NO	Norway
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	SD	Sudan
CF	Central African Republic	KP	Democratic People's Republic of Korea	SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
DE	Germany, Federal Republic of	LU	Luxembourg	TG	Togo
DK	Denmark			US	United States of America

- 1 -

TITLE OF THE INVENTION

NOVEL MATERIALS AND METHODS FOR GUIDED TISSUE REGENERATION

BACKGROUND OF THE INVENTIONField of the Invention

The present invention relates to novel materials, compositions and methods for guided tissue regeneration in animals including humans.

Description of Related Art

Regeneration or healing of diseased and injured tissue is of major practical clinical importance. With respect to periodontal and long bone defects especially, one of the most significant impediments to satisfactory regeneration is invasion of a wound site by tissue with little or no potential to differentiate into the appropriate skeletal or connective tissue type. Migration and proliferation of epithelial cells into a skeletal wound site, for example, can prevent repopulation by osteogenic cells which migrate more slowly.

Use of membranes to mechanically retard or prevent epithelial cell migration and to guide osteogenic cells onto the healing surface is one reported approach to this problem. As the following discussion illustrates, however, such methods suffer from limitations which restrict their effectiveness.

Information Disclosure Statement

5 In accordance with the requirements of 37 CFR §1.56, the following are concise explanations of documents known to Applicants or their attorney, submitted in accordance with 37 CFR §§1.97 and 1.98.

Applicants will submit hereafter on form PTO-1449 a listing of these documents in accordance with 37 CFR §1.98, together with copies of the listed documents.

10 Applicants do not waive any rights to appropriate action to establish patentability over any of the listed documents should they be applied as references against the claims of the present application.

15 This statement should not be construed as a representation that more material information does not exist or that an exhaustive search of the relevant art has been made.

20 Consideration of these documents, and making the same of record in the prosecution of the present application upon submission of the form PTO-1449 and copies of the documents listed therein, are respectfully requested.

25 One approach to guided tissue regeneration has been to cover a wound site with a passive non-resorbable membrane. Typically, this membrane will be composed of a biocompatible inert material, which is sutured in place. GoreTextm (polytetrafluoroethylene) and Millipore filters are two materials which have been used as membranes. The greatest drawback to the use of such inert materials is that they must be surgically removed. This procedure, of course, disrupts the healing process, and can lead to unwanted infection of the wound site.

30 Thus, United States Patent No. 4,752,294 describes an element for controlled growth of tissue which can may be used for attaching a prosthesis to the body or for controlled

regeneration of tissue around teeth. The element is stated to be a passive non-resorbable biocompatible material having perforated walls and cavities of defined size, and to function by mechanically directing growth of epithelial and connective tissue.

Other similar reports using passive non-active membranes have appeared. Thus, in beagle dogs having natural periodontitis, the use of Gore-Textm membranes for guided tissue regeneration is described in regeneration of furca bone (Niederman *et al.*, *J. Dent. Res.* 66(*Spec. Issue Mar.*):281, Abstr. 1392 (1987)), and debrided mandibular molars and premolars (Caffesse *et al.*, *J. Dent. Res.* 66(*Spec. Issue Mar.*):280, Abstr. 1391 (1987)). Subgingivally placed Gore-Textm membranes were stated to promote formation of new connective tissue attachment by preventing dentogingival epithelium and gingival connective tissue from interfering with cementum formation on surgically exposed and planed root surfaces in monkeys (Gottlow *et al.*, *J. Dent. Res.* 66(*Spec. Issue Mar.*):281, Abstr. 1394 (1987)). Pontoriero *et al.*, *J. Dent. Res.* 67(*Spec. Issue Mar.*):272, Abstr. 1277 (1988), covered Class II furcation defects in mandibular molars with Gore-Textm membranes which were removed 1-2 months later. They reported a reduction in probing depth after 3 and 6 months in treated *versus* untreated patients.

Aukhil *et al.*, *J. Dent. Res.* 66(*Spec. Issue Mar.*):281, Abstr. 1396 (1987), attached pieces of Millipore filter to the crowns of premolars and molars of monkeys from which three mm of the buccal and interproximal bone had been removed. No differences in ³H-thymidine labeling were observed in teeth with or without the Millipore filter barriers in the periodontal ligament region apical to the wound site.

Gottlow *et al.*, *J. Clin. Periodontol.* 11:494-503 (1984), describe the placement of Millipore or Gore-Textm membranes over denuded root surfaces of surgically created defects in

monkeys. After three months of healing, the animals were sacrificed. The authors report new cementum with inserting collagen fibers in nine of nine test roots and five of six control roots.

5 Gottlow et al., *J. Clin. Periodontol.* 13:604-616 (1986),
used a teflon membrane to guide tissue regeneration in human
patients with advanced periodontal disease. After three
months of healing with the membranes in place, they were
10 removed in a second surgical procedure. Another three months
healing period preceded final examination of treated teeth.
The authors state that the amount of newly formed fibrous
attachment "was found to vary considerably from one tooth to
the next tooth."

15 Dahlin et al., *Plastic and Reconstructive Surg.*,
81(5):672-676 (1988), describe a method of mechanically
preventing connective-tissue ingrowth by a membrane technique.
A porous (0.45 μ m) polytetrafluoroethylene membrane was used
to cover experimentally produced through-and-through osseous
20 mandibular defects (5 mm diameter) in rats. Healing was
observed on membrane-covered defects where membrane displace-
ment had not occurred. The chemically inert Teflon membranes
were not removed prior to sacrifice. The authors state that
"[f]rom the clinical point of view, it might seem more
25 advantageous to use some type of membrane which is eventually
resorbed into the tissue," but that "this could introduce
problems such as local inflammatory response with phagocytic
activity and the need to maintain proper timing between
completion of bone regeneration and degradation of the
membrane."

30

In contrast to the use of inert synthetics, another
approach to guided tissue regeneration has involved passive
biodegradable membranes such as collagen. The major short-

coming of such methods, as suggested by Dahlin *et al.*, *supra*, is the high incidence of inflammatory response.

5 Blumenthal, J. *Periodontol.* 59(12):830-836 (1988), used commercial membranes, prepared from bovine collagen cross-linked with glutaraldehyde, to exclude gingival connective tissue and epithelium and to guide new connective tissue attachment in induced interproximal root surface defects in dogs. The authors report that, by two weeks, the collagen membrane was closely adhered to the cemental surface, 10 preventing contact with gingival connective tissue, the fibers of which were sparse, disorganized, and obliterated in areas by the chronic inflammatory infiltrate. By eight weeks, the membrane was almost completely resorbed, and a moderate chronic inflammatory response was present, although no apical epithelial migration occurred. New connective tissue 15 attachment to cementum was evident, and the connective tissue was vascularized with mild chronic perivascular inflammatory infiltration. At 12 weeks, the connective tissue attachment had matured, with a functional, dense fiber orientation and decreased vascularity and inflammatory cells. 20

Pitaru *et al.*, *J. Dent. Res.* 65(Spec. Issue B):822, Abstr. 870 (1986), covered surgically created buccal root surface defects in mongrel dogs with purified rat collagen membranes. Histological examination ten days later revealed 25 that the membranes prevented apical migration of oral epithelium.

Magnusson *et al.*, *J. Dent. Res.* 65(Spec. Issue B):822, Abstr. 866 (1986), compared inert Millipore filters with biodegradable polylactic acid membranes for guided tissue 30 regeneration in surgically created buccal root defects in dogs. The authors state that there was significantly less new cementum attachment with Millipore filters than with the polylactic acid membranes, and that recession was observed with the filters.

Fleisher *et al.*, *J. Dent. Res.* 66(Spec. Issue Mar.):281, Abstr. 1393 (1987), used Vicryl (polygalactin-910), a slowly resorbable, biocompatible, tightly woven mesh graft, to guide tissue regeneration in induced buccal defects in dogs. The authors report that the Vicryl, which has been used in neurosurgery as a dural substitute, inhibited connective tissue and epithelial tissue participation in healing, and was totally absorbed at six weeks.

Terranova *et al.*, *J. Dent. Res.* 66(Spec. Issue Mar.):280, Abstr. 1390 (1987), describe the use of a reconstituted biodegradable basement membrane to inhibit epithelial cell migration to dentin. The authors note that biochemical modulation of the dentin surface has been shown to modulate epithelial cell adherence and growth. Gingival epithelial cells penetrated a type I collagen barrier when epithelial cell specific chemoattractants (LM or EGF) were applied to the dentin surface. Incorporation of type IV collagen and laminin into the type I collagen matrix, to produce a reconstituted basement membrane, prevented epithelial cell penetration. The authors also report that gingival fibroblasts migrate through a type I collagen barrier when fibroblast specific chemoattractants (PDGF) were placed on the dentin surface. By contrast, gingival fibroblast and epithelial cell migration between adjacent dentin surfaces was prevented by interposing the reconstituted basement membrane.

West *et al.*, *J. Dent. Res.* 67(Spec. Issue Mar.):273, Abstr. 1281 (1988), describe the use of a bovine-tendon-derived absorbable collagen membrane to guide tissue regeneration in non-furcated intrabony human periodontal defects. It is stated that probing measurements were reduced from zero to four mm in treated defects, and from zero to two mm in non-treated defects.

Aukhil *et al.*, *J. Periodontol.* 57(12):727-734 (1986), attempted to combine the properties of an inert barrier and a

biodegradable matrix into a single membrane. These authors used a biocomposite of an ultrathin, semipermeable silicone membrane mechanically bonded to a flexible knitted nylon fabric and coated with hydrophilic collagen peptides (trade-named "Biobrane") as a physical barrier to prevent contact of oral epithelium and gingival connective tissue with curetted root surfaces of beagle dogs with periodontal disease. This membrane, with the flat surface (silicone membrane side) facing the tooth and the collagen-coated network facing the flaps to be replaced, was attached to the teeth with composite resin. After five weeks, however, the membranes were removed because of the progressive recession of the flap margins, which resulted in increased plaque accumulation. The authors state: "The fibrous collagen-coated network of the membrane was a retentive factor for plaque and debris." Mechanical resistance and bleeding were encountered during removal of the membrane. The authors suggest that the Biobrane interfered with the blood supply to the gingival margin, resulting in progressive recession of the flap margins. Where the Biobrane was incompletely removed, it became "surrounded by chronic inflammatory cells and giant cells."

Another reported approach to wound healing is the use of implants or grafts. The general objective of such implants is to provide a base for cell migration and growth, rather than to guide migration or exclude certain cells. Such methods typically involve injection of a collagen gel into the wound site. Other substances, such as demineralized bone extracts, may be mixed throughout the gel. Implant techniques suffer from the same potential for adverse inflammatory response as do collagen gel membrane techniques.

Levy et al., *J. Periodontol.* 52:303-306 (1981), implanted a calf skin collagen-calcium phosphate gel into surgically induced two-walled bony defects in dogs and monkeys. The

authors report that no significant bone height regeneration occurred, although connective tissue formation and re-epithelialization were observed. Moderate to dense inflammatory response was present adjacent to all root surfaces proximal to the resorbing gel.

5 Busschop *et al.*, *J. Clin. Periodontol.* 10:399-411 (1983), used a lyophilized, allogenic dura matter material (tradenamed "Lyodura") as an implant material in interdental bony craters (two-wall lesions) in humans. The authors report gains in probing attachment level and greater reduction in probing pocket depth as compared to non-implanted controls. It is stated that the Lyodura seemed to act as a barrier against epithelial downgrowth and inflammatory cell infiltration. The Lyodura material is purified dura matter cerebri taken from human corpses.

10 Yaffe *et al.*, *J. Periodontol.* 55(11):623-628 (1984), describe the use of an enriched collagen solution (ECS) for treatment of intrabony defects in dogs. ECS was prepared from a 0.2% acid soluble native dog skin collagen enriched with a variety of cell nutrients. This solution was injected into three-wall intrabony defects. After four weeks of healing, epithelium downgrowth was arrested and new cementum and new alveolar bone formation was reported in ECS-treated defects.

20 Blumenthal *et al.*, *J. Periodontol.* 57(2):84-90 (1986), describe a combined grafting technique employing a bovine dermal collagen gel (tradenamed "Zyderm") and freeze-dried demineralized autolyzed antigen-extracted allogeneic (AAA) bone implant. A 1 mm thick film of collagen gel alone was injected into the base of surgically induced periodontal defects in mongrel dogs. Rehydrated AAA bone mixed with the collagen gel was then placed into the defects, contiguous to the collagen film, after which the mucoperiosteal flaps were closed. The authors report that the combined collagen-AAA bone graft specimens showed the greatest degree of new

attachment at 4, 10, 16 and 24 weeks, as compared to untreated controls, non-implanted controls, and specimens treated with AAA bone only. The authors state that the collagen gel appeared to inhibit apical migration of the junctional epithelium by providing a physical barrier against epithelial cell movement along the root. It is also stated that the AAA bone acted as a potent osteoinducer.

Porous hydroxyapatite is another substance which has been used as an implant. El Deeb et al., *J. Dent. Res.* 65(Spec. Issue B):822, Abstr. 864 (1986), used porous hydroxyapatite Interpore 200 (PHI) as a bone substitute to fill three wall iliac crest bone defects in monkeys. Histological examination revealed surrounding bone, muscle and connective tissue penetration of all implants. Kenney et al., *J. Dent. Res.* 65(Spec. Issue B):822, Abstr. 867 (1986), used PHI to fill class II furcations in thirteen human patients. The authors report reduced pocket depths, increased attachment level gains, and increased vertical and horizontal bone level gains as compared to unfilled control defects.

The preceding discussion illustrates that considerable efforts have been expended to develop materials and methods for guided tissue regeneration. These materials and methods, however, have suffered from various drawbacks. As a result, there continues to be a need for an improved membrane for use in guided tissue regeneration.

SUMMARY OF THE INVENTION

There have now been invented novel materials and compositions useful in guiding tissue regeneration, and methods of using these materials and compositions. The novel membranes of the invention are characterized in being of a biodegradable material which is coated on one or more surfaces with one or more bioactive substances. The biodegrad-

able bioactive membranes of the present invention are further characterized in that the specific bioactive substance or substances is chosen with reference to the particular tissue or cell type which it is desired to enhance or inhibit. Further, the choice of coating one or both sides of the bioactive biodegradable membranes of the invention is made with reference to the manner in which tissue regeneration may proceed with best therapeutic effect.

Thus, in one illustrative embodiment, there is provided a composition for guided tissue regeneration comprising a bioactive biodegradable membrane comprising a first and a second side, wherein said first side of said membrane comprises one or more biologically active substances not present on said second side.

In another embodiment, the present invention provides for a composition for guided tissue regeneration comprising a biodegradable bioactive membrane comprising two sides, wherein a first and a second side of said membrane each comprises one or more biologically active substances, and wherein at least one of said one or more biologically active substances on said second side is not present on said first side.

Yet additional embodiments comprise the preceding compositions, wherein said one or more biologically active substances is selected from the group consisting of drugs, peptides and proteins. Biologically active substances (BAS) may include hormones and growth factors. Further, biologically active substances according to the invention may be selected from, among others, epidermal growth factor, insulin-like growth factor, platelet derived growth factor, fibronectin, angiogenesis factor, and bone morphogenic protein, as well as those substances listed in Table II.

Additional embodiments comprise the preceding compositions, wherein the composition further permits the controlled release of BAS. In one embodiment, the release of BAS is

controlled by covering at least part of the BAS-coated side of the membrane with a resorbable membrane layer. In this embodiment, BAS will be immediately available in areas of the membrane not covered by the resorbable membrane layer, whereas BAS availability in other areas of the membrane will require resorption of the resorbable membrane layer.

In another embodiment, a resorbable membrane layer covering some or all of the BAS-coated side of the membrane may contain varying amounts of a component which may enhance or inhibit the rate of resorption, thereby controlling the rate at which BAS is released.

In a further embodiment, the BAS may be incorporated into a membrane comprising one or two layers.

The rate of BAS release may be further controlled by covering at least part of the BAS-coated membrane with a second resorbable membrane layer having a varying degree of cross-linked components. The rate at which the membrane is resorbed will vary with the extent of cross-linking, thereby varying the rate of release of BAS.

In order to reduce the incidence of adverse inflammatory responses reported with the use of the present novel biodegradable bioactive membranes, the compositions of the invention may additionally comprise an anti-microbial or other inhibitory agent on one or both sides, as appropriate for a given therapeutic application.

Non-limiting examples of suitable biodegradable membrane components according to the invention include polylactic acid, polyethylene oxide/polylactic acid (PELA), collagens, laminin and fibronectin.

In another aspect, there are provided methods of guiding regeneration of tissue in animals, including humans, comprising applying in close apposition to said tissue compositions such as those described above. Non-limiting examples of tissues which may be appropriate for guided tissue regenera-

tion according to the methods of the invention include epithelial tissue, bone tissue, ophthalmic tissue, vascular tissue and endothelial tissue.

5 In yet another aspect, there is provided according to the invention a process for preparing a composition for guided tissue regeneration, comprising conjugating to one or more sides of a biodegradable membrane a biologically active material.

10 In the above embodiments, it may be desirable to affix the membrane to the tissue. The means for affixing the membrane will depend upon, for example, the site of placement of the membrane and the size of the membrane. For some embodiments the membrane may be affixed by suturing. In other
15 embodiments, it may be desirable to affix the membrane via an adhesive material attached to or incorporated in part or all of the membrane.

DESCRIPTION OF THE FIGURE

20 Figure 1 is a representation of a biodegradable bioactive membrane having a bioactive substance. Some of the bioactive substance is covered by a resorbable membrane layer composed of one or more resorbable membranes assembled in a stepwise
25 fashion.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

30 In the following description, reference will be made to various publications and other materials. Publications and other materials to which reference is made are incorporated herein by reference in their entireties as though set forth herein in full.

General principles of biocompatible biodegradable membrane construction are set forth, for example, in

Gunasekaran et al., *Biomat., Art. Cells, Art. Org.* 16:771-784 (1988); and Hayward et al., *Biomaterials* 1984 5:135-142 (1984). General surgical procedures involving the use of guided tissue regeneration are set forth, for example, in the
5 "GoreTextm Periodontal Material Training Manual," W.L. Gore & Assocs., Inc., pp. 10-33 (1988); Becker et al., *J. Periodontol.* 58:819-826 (1987); and Gottlow et al., *J. Clin. Periodontol.* 13:604-616 (1986).

By "guided tissue regeneration" according to the present
10 invention is meant the placement of a biodegradable membrane which is coated on one or more surfaces with one or more BAS at or in close apposition to the site of tissue loss or deficit, by means of which the migration, proliferation, or maturation of certain tissue or cell types may be enhanced or,
15 if desired, inhibited. Such enhancement or inhibition will be understood by those of skill to be included within the definition of tissue guidance for the purposes of the present invention. Instead of or in addition to the BAS coating, the biodegradable membrane may have one or more BAS incorporated
20 within it.

By "biodegradable membrane" is meant generally any relatively thin layer of tissue, such as that which may cover a surface, line a cavity, or divide a space or organ, which is susceptible of degradation by biological processes. Such
25 membranes may be derived from natural or synthetic sources, or may comprise a combination of natural and synthetic elements. It will be appreciated, of course, that biodegradable membranes according to the present invention will preferably also be biocompatible, i.e., they will not have uncontrolled or uncontrollable inherent toxic or injurious effects upon
30 biological functions.

The composition of biodegradable membranes according to the present invention will vary, depending upon the type of tissue(s) which it is desired to regenerate. Such membranes

could include, but not be limited to, collagen membranes prepared commercially from a molecular solution of purified bovine collagen, cross-linked with glutaraldehyde under sterile conditions. Such membranes have been shown to successfully guide connective tissue attachment and generally last between 6 and 8 weeks before being resorbed by the host (Pitaru *et al.*, *J. Dent. Res.* 65(Spec. Issue B):822, Abstr. 870 (1986); Blumenthal, *J. Periodontol.* 59(12):830-836 (1988)).

It will be recognized that the rate at which the biodegradable membranes of the present invention are resorbed is an important factor in achieving an optimal therapeutic result. Membranes which are resorbed too quickly will provide insufficient tissue guidance. Further, overly rapid resorption rates will interfere with the desirable therapeutic effects of the bioactive substances which coat the first or second side of such membranes.

On the other hand, resorption rates which are too slow may interfere with tissue regeneration, in that, among other possible drawbacks, the membrane may act as a hindrance to proliferation or maturation of desirable cell types. Because of the nature of the bioactive, biodegradable membranes of the present invention, however, it will be generally preferable to design membranes having resorption rates which are slightly too slow, rather than too fast. Of course, those of skill will recognize that desirable resorption rates will vary as a function of, *inter alia*, the rate of migration, proliferation and maturation of the predominant cell type involved. Further, it will be evident that the composition of the biodegradable membranes of the invention will be chosen and the membranes assembled with reference to the particular target tissue, with the exercise of no more than routine skill.

Another suitable biodegradable membrane may be composed of polylactic acid. Polylactic acid is a biodegradable ester polymer, synthesized from cyclic lactides. It contains no peptide linkages. Polylactic acid membranes are available commercially, and have been used successfully for guided tissue regeneration and as a surgical mesh to treat facial deformities, arterial defects, dural defects and dental surgical defects (Kulkarni *et al.*, *Arch. Surg.* 9:839 (1966); Kulkarni *et al.*, *J. Biomed. Mater. Res.* 5:169-181 (1971); Magnusson *et al.*, *J. Dent. Res.* 65(Spec. Issue B):822, Abstr. 866 (1986)).

A variant of the polylactic acid membrane is the polyethylene oxide/polylactic acid (PELA) membrane. This membrane is a co-polymer of polylactic acid and may be more efficacious in certain applications, since it exhibits a relatively more rapid resorption rate (Younes *et al.*, *Biomat., Art. Cells, Art. Organs* 16:705-719 (1988)).

A preferred biodegradable membrane for use in accordance with the present invention will be composed, in major part, of collagen. Several different collagenous proteins have been isolated from connective tissues. The collagen family of proteins presently comprises 11 different types, designated types I-XI. Different collagen types often are characteristically associated with different natural connective tissues. Accordingly, it will be evident to those of skill that the composition of a collagen-based biodegradable bioactive membrane may be adjusted with respect to the type and relative proportion of collagen used, so as to provide an optimal membrane for guided tissue regeneration of a given tissue.

The fibril-forming collagens are types I, II and III. Of these, type I is most abundant. Type II is the main collagenous protein of cartilage. Type III accompanies type I in different ratios in almost all tissues. Type IV collagen differs markedly in structure from the fiber-forming col-

lagens, forming a non-fibrillar network, and is found in basement membranes.

5 The structure and function of different collagen types is described, for example, in R. Mayne and R.E. Burgeson, eds., "Structure and Function of Collagen Types," Academic Press, Inc., Orlando, Florida (1987). All collagens have in common a triple-helical domain, which is combined differently with globular and non-helical structural elements. Collagen aggregates are stabilized by intermolecular covalent bonds located at specific regions along the triple helix. These domains also are important for degradation of the fibrils by collagenase, and for the interaction of collagen and other matrix constituents. These sites are characterized by low proline and hydroxyproline content.

10 The glycoprotein fibronectin can serve as a mediator between collagen and the cell surface. As such, it is another constituent which those of skill will recognize as a desirable component of biodegradable membranes according to the present invention, for many tissue applications. Fibronectin has particularly high affinity for denatured collagens, Jilek et al., *Hoppe-Seyler's Z. Physiol. Chem.* 359:247-250 (1978), and contains binding sites for collagen and for the cell surface. Fibronectin is reviewed, for example, in Yamada, *Ann. Rev. Biochem.* 52:761-799 (1983), and Yamada et al., *J. Cell Biochem.* 28:79-97 (1985). Plasma fibronectin shares antigenic expression and forms precipitant lines of identity on double immunodiffusion against polyclonal antisera prepared to any other fibronectin species, and has been described as a form of fibronectin distinct from extracellular or cell-associated fibronectin. See, J. McDonagh, ed., "Plasma Fibronectin: Structure and Function," Marcel Dekker, Inc., New York (1985). Those of skill will recognize that plasma fibronectin may be a desirable component of the biodegradable membranes according to the present invention. Alternatively, plasma fibronectin

may be coated on the surface of a biodegradable membrane as a bioactive substance in keeping with the present invention. The action of plasma fibronectin in wound repair is described in Clark, et al., in, J. McDonagh, ed., "Plasma Fibronectin: Structure and Function," Marcel Dekker, Inc., New York, pp. 197-262 (1985).

Variations in the macromolecular structure of collagen types are believed to modify the biomechanical properties of the extracellular matrix to the various physiological functions of different connective tissues. The ability of the fiber-forming collagens to vary their macromolecular organization is not enough to build stabilizing scaffolds for all of the different extracellular matrices. To introduce higher variability, triple-helical elements are believed to be combined with globular domains on the one hand, and on the other hand, the length and some structural features of the triple-helical domain itself often may be altered. For example, type IV collagen is found only in basement membranes. The molecules of type IV collagen do not form fibrils, but form instead a network in which they are connected to one another via their like ends. Flexibility is introduced by frequent interruption of the triple helix by non-helical areas. Also, the C-terminal globular domain of the type IV collagen molecule is not removed, but is used as an integral part of the final macromolecular structure of the molecule. In this respect, type IV collagen differs from the fiber-forming collagens. R. Mayne and R. E. Burgeson, *supra*.

Table I summarizes the major types of collagens, their molecular and supramolecular structure, and localization within tissue types. Review of Table I demonstrates that certain collagen types are predominantly localized in particular tissues. Those of skill will recognize that, in constructing biodegradable membranes according to the present invention, the choice of collagen types will be dependent

upon the target tissue for which regeneration is desired. For example, type VI collagen is particularly rich in the cornea. Type VII is the major and perhaps sole component of anchoring fibrils, and cornea demonstrates the most extensive anchoring fibril network visualized thus far. Type VIII collagen, also known as endothelial collagen, is secreted by most endothelial cells in culture. Highest type VIII levels are observed in rapidly proliferating or migrating cells plated at low density. Type VIII collagen also is found in high concentrations in Descemet's membrane, which is the basement membrane which separated corneal endothelial cells from corneal stroma. Thus, as a non-limiting example of the preparation of a biodegradable membrane according to the present invention, a collagen-based membrane intended for use in guiding tissue regeneration of corneal tissue might contain relatively greater amounts of type VI, type VII, and type VIII collagens. These collagen types, however, might not be included in the preparation of a biodegradable membrane according to the present invention which was intended for use in guiding regeneration of skin, tendon, muscle, bone, etc., which might contain relatively larger proportions of type I and type III collagens.

TABLE I

Major collagen types, their molecular and supra-molecular structure, and localization within tissue types.

Type	Chains	Molecular structure	Supramolecular structure	Localization
I	$\alpha 1(I)$, $\alpha 2(I)$	300 nm	67-nm banded fibrils	Skin, tendon, bone, etc.
II	$\alpha 1(II)$	300 nm	Small 67-nm banded fibrils	Cartilage, vitreous humor
III	$\alpha 1(III)$	300 nm	Small 67-nm banded fibrils	Skin, muscle, etc.
IV	$\alpha 1(IV)$, $\alpha 2(IV)$	390 nm C globular domain	Nonfibrillar network	All basement membranes
V	$\alpha 1(V)$, $\alpha 2(V)$, $\alpha 3(V)$	300 nm N globular domain	Small fibers	Most interstitial tissues
VI	$\alpha 1(VI)$, $\alpha 2(VI)$, $\alpha 3(VI)$	150 nm N+C globular domains	Microfibrils, 100 nm banded fibrils	Most interstitial tissues
VII	?	450 nm	Dimer	Anchoring fibrils
VIII	$\alpha 1(VIII)$?	?	Some endothelial cells
IX	$\alpha 1(IX)$, $\alpha 2(IX)$, $\alpha 3(IX)$	200 nm N globular domain	?	Cartilage
X	$\alpha 1(X)$	150 nm C globular domain	?	Hypertrophic and mineralizing cartilage
XI	$\alpha 1(XI)$, $\alpha 2(XI)$, $\alpha 3(XI)$	300 nm	Small fibers	Cartilage

(Table I is derived from R. Mayne and R.E. Burgeson, eds., "Structure and Function of Collagen Types," Academic Press, Inc., Orlando, Florida, p. 2 (1987)).

By contrast, a biodegradable collagen-based membrane, according to the invention, which was intended for use in regeneration of hyaline cartilage, might contain a relatively higher proportion of type IX collagen. This collagen is a proteoglycan, predominantly present in the pericellular matrix which immediately surrounds chondrocytes. Type XI collagen might also be included for such purposes according to the present invention. Where an object of guided tissue regeneration was regeneration of long bone or skeletal tissue, preparation of a biodegradable membrane according to the invention could preferably include relatively larger amounts of type X collagen. It is known, for example, that accumulation of type X collagen precedes calcification of tissue. It is the major collagen type synthesized in most mature chondrocytes involved in endochondral bone formation. Thus, type X collagen bridges the spatial and temporal transition from cartilage to bone.

Another possible component of biodegradable membranes according to the present invention is laminin. Laminin is a multi-functional protein with diverse biological activities. Like fibronectin, it is believed to influence cell adhesion, growth, morphology, differentiation, migration, and agglutination, as well as the assembly of the extra-cellular matrix. Laminin primarily affects cells of epithelial origin, and the response varies depending upon the cell. Laminin is a large glycoprotein present in all basement membranes, where it is the most abundant constituent. It binds to various components of basement membrane and probably links these to one another to form an integrated complex. A specific cell surface receptor to laminin has been identified. Laminin and its biological activities are described in Kleinman et al., *J. Cell Biochem.* 27:317-325 (1985). Laminin binds to matrix components, including type IV collagen, heparin sulfate proteoglycan, entactin, and nidogen, which those of skill will

recognize as other elements which it may be desirable to incorporate into the biodegradable bioactive membranes of the invention. Laminin promotes attachment of various epithelial cells to type IV collagen-coated substrates. Unlike fibronectin, laminin is able to bind either to the cell surface or to collagen, although laminin appears to be most effective as an attachment protein for epithelial cells when bound to type IV collagen.

The importance of fibronectin in the wound-healing process is described by Grinnell, *J. Cell Biochem.* 26:107-116 (1984). In cutaneous wound healing particularly, fibronectin modulates the function of platelets, neutrophils, monocytes, fibroblasts, endothelial cells, and keratinocytes. The plasma enzyme transglutaminase (factor VIII) cross-links the fibrin polymer which is a major structural component of the blood clot that fills a skin wound defect. Thus, it will be evident that factor VIII, which also covalently links fibronectin to the fibrin polymer, may be a desirable bioactive substance for use in the biodegradable membranes of the present invention.

Elastin is an important component of the extracellular matrix in tissues that undergo repeated elastic recoil. It thus may be an especially desirable component of the biodegradable bioactive membranes of the invention when guided regeneration of such tissues is indicated. Together with glycoprotein microfibrils, elastin forms extracellular fibers that provide resiliency essential for normal tissue function. Cell types that synthesize elastin include smooth muscle cells, fibroblasts and myofibroblasts, endothelial cells, and chondrocytes in elastic cartilage. The elastic properties of many tissues of the vertebrate body, such as the lung and larger arteries, are due mainly to the presence in the extracellular matrix of elastic fibers composed primarily of the protein elastin. Individual polypeptide chains within the mature elastin fibers are covalently connected by cross-

linkages derived from the oxidation of lysine residues. See, Mecham *et al.*, "Extracellular Matrix Promotes Elastogenic Differentiation in Ligament Fibroblasts," in, A.H. Reddi, ed., "Extracellular Matrix: Structure and Function," Alan R. Liss, Inc., New York, pp. 383-392 (1985); Pasquali-Ronchetti *et al.*, in A. Ruggeri and P.M. Motta, eds., "Ultrastructure of the Connective Tissue Matrix," Martinus Nijhoff Publishers, Boston, pp. 126-139 (1984); and Serafini-Facassini, in A. Ruggeri and P.M. Motta, eds., "Ultrastructure of the Connective Tissue Matrix," *supra*, pp. 140-150.

Proteoglycans are a diverse group of heterogeneous macromolecules that are most abundant in the extracellular matrix of connective tissues. Proteoglycans contain core proteins to which one or more glycosaminoglycan side chains are covalently attached. Glycosaminoglycans are linear anionic polysaccharides with repeating disaccharide units containing a hexosamine residue and usually, but not always, a hexuronic acid residue. Proteoglycans, and especially chondroitin sulfate, are found in high concentrations in cartilage. Most of the proteoglycans within the cartilage matrix seem to be present as aggregates. Proteoglycans are central to the proper functioning of cartilage, which is to be able to reversibly absorb loading forces. There are experimental data which suggest that proteoglycans play an active role in the mineralization process. Thus, those of skill will appreciate that proteoglycans may be desirable constituents of biodegradable bioactive membranes according to the present invention, especially with respect to guided regeneration of cartilage tissue. Proteoglycans are reviewed, for example, by Kuttner *et al.*, *J. Cell Biochem.* 27:327-336 (1985); and T.N. Wight and R.P. Mecham, eds., "Biology of Proteoglycans," Academic Press, Inc., Orlando (1987).

It will be appreciated that the various components may be incorporated into the biodegradable bioactive membranes of the

present invention in their mature forms, as well as in known precursor forms, and further, that synthetic analogues of these components also may be advantageously used. Moreover, those of skill will recognize that, under appropriate circumstances, it may be desirable to employ such components as bioactive agents to coat one or more surfaces of the membrane, rather than as constituents of the membrane matrix itself.

General principles of extracellular matrix structure and function in development and disease are set forth, for example, in A.H. Reddi, ed., "Extracellular Matrix: Structure and Function," Alan R. Liss, Inc., New York, pp. 383-392 (1985); and A. Ruggeri and P.M. Motta, eds., "Ultrastructure of the Connective Tissue Matrix," Martinus Nijhoff Publishers, Boston (1984). Principles guiding the relationship between cell movement and extracellular matrices are set forth, for example, in R. Porter and J. Whelan, eds., "Basement Membranes and Cell Movement," Pittman, London (1984).

An important aspect of the present invention is that the biodegradable bioactive membrane, comprising two sides, will have coated onto at least one of said sides one or more bioactive agents. Incorporation of such bioactive agents onto the surface of the biodegradable bioactive membranes of the present invention results in selective enhancement or, if desired, inhibition of host or foreign cell or tissue migration, proliferation, or maturation which has not previously been possible. Those of skill will recognize that the selection of bioactive agents for use according to the present invention will be made with reference to the particular types of host or foreign cell or tissues which it is desired to enhance or inhibit.

The choice of bioactive agents will thus be made by those of skill, keeping in mind general principles of cell migration, proliferation, and maturation. The principal bioactive

agents which may be employed in accordance with the present invention include, but are not limited to, drugs, hormones and peptide growth factors.

5 By "drugs" is meant any chemical compound that may be used on or administered to humans or animals as an aid in the diagnosis, treatment, or prevention of disease or other abnormal condition, for the relief of pain or suffering, or to control or improve any physiologic or pathologic condition. It will be appreciated that drugs useful in the present invention may act to enhance or inhibit host or foreign cellular or tissue function, and that the choice of such drugs will be made in accordance with generally accepted principles of clinical therapeutics. Such principles are set forth, for example, in A. G. Gilman et al., eds., "Goodman and Gilman's The Pharmacological Basis of Therapeutics," 7th Ed., MacMillan Publishing Co., New York (1985).

10 In a preferred embodiment of the invention, a first side of the biodegradable bioactive membrane will be coated with one or more agents which enhance the migration, proliferation or maturation of a particular cell type. This first side will be placed at the site of a wound or defect such that it faces said wound or defect, in order to achieve the optimal enhancing effects of the agent or agents which coat this first side. A second side of said membrane will be uncoated, or, preferably, coated with one or more inhibiting agents. Such agents might include, for example, anti-microbial agents, in order to reduce the incidence of undesirable inflammation and infection. Suitable anti-microbials which may be used in accordance with the invention will be selected by those of skill in accordance with accepted principles of clinical anti-microbial treatment. Thus, the general goal in choosing an anti-microbial is to select a drug or other agent which is selectively active for the most likely infecting micro-organism(s), and that has the least potential to cause

toxicity or allergic reactions in the individual being treated. Among the anti-microbial agents which may be selected for use in accordance with the invention may be mentioned the sulfonamides, trimethoprim-sulfamethoxazole, penicillins, cephalosporins, and other β -lactam antibiotics, aminoglycosides, tetracyclines, chloramphenicol, erythromycin, and other anti-bacterial agents, as well as anti-fungal and anti-viral agents. These, and other appropriate agents, and their selection and clinical use, are described in, for example, A. G. Gilman *et al.*, eds., "Goodman and Gilman's The Pharmacological Basis of Therapeutics," 7th Ed., MacMillan Publishing Co., New York (1985), particularly in Section XII, "Chemotherapy of Microbial Diseases."

The second side of the biodegradable bioactive membrane of the invention may, in another embodiment, be coated with an enhancing agent. This agent may be the same as that which is used to coat the first side of the membrane. In a preferred embodiment, however, it will be a different agent, and may be chosen with the object of enhancing a different host cell or tissue type than that which is the object of the first agent coating the first side of the membrane. In this manner, it will be possible according to the invention to differentially enhance and guide regeneration of different cell or tissue types on each side of the membrane.

The second side of the membrane may be coated with factors to regulate epithelial growth. One example of such a factor is EGF, and other examples will be apparent to one of skill in the art. The second side may be coated with a fibroblast proliferation regulator, an example of which is TGF α or β .

Drugs and other bioactive agents used in accordance with the present invention may be coated on the surface of the biodegradable bioactive membranes of the invention by any known methods, including the use of cross-linking molecules

such as proteins. The enzyme factor VIII is one non-limiting example of a protein cross-linking molecule which may be employed in this regard. Cross-linkage also may be performed in some systems using irradiation at low temperatures. Other techniques for coating one or both surfaces of the biodegradable bioactive membranes of the present invention will be determined by those of skill with reference to the particular agent, the composition of the membrane, and other factors, with the exercise of routine skill. It will thus be appreciated that the term "coated" as used herein refers broadly to any method or process which allows the achievement of the desired result as herein described.

It will be recognized that drugs and other active substances may be employed in the form of pharmaceutical compositions, and, of course, that many combinations of active agents may be beneficially used on one or both sides of the biodegradable bioactive membranes of the invention. Accordingly, such pharmaceutical compositions may be administered to any animal which may experience the beneficial effects of the compounds of the invention. Foremost among such animals are humans, although the invention is not intended to be so limited.

The dosage of the active agents administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. In addition to the pharmacologically active compounds, the pharmaceutical preparations may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, for example, by means of conventional mixing, granulating, dissolving, or lyophilizing processes. Thus,

pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding the resulting mixture and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary.

5 Suitable excipients are, in particular, fillers such as saccharides, for example lactose or sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, for
10 example, maize starch, wheat starch, rice starch, potato starch, gelatin, tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents may
15 be added such as the above-mentioned starches and also carboxymethyl-starch, cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are, above all, flow-regulating agents and lubricants, for example, silica, talc, stearic acid or
20 salts thereof, such as magnesium stearate or calcium stearate, and/or polyethylene glycol.

TABLE II

Growth Factors, Hormones, and Other Growth-Regulating Agents of Exogenous Cellular Origin

Well characterized growth factors

Insulin-like growth factor I (IGF-I) or somatomedin C
 Insulin-like growth factor II (IGF-II), somatomedin A, or multiplication-stimulating activity (MSA)
 Epidermal growth factor (EGF) or urogastrone (URO)
 Fibroblast growth factor (FGF) or fibroblast-derived growth factor (FDGF)
 Nerve growth factor (NGF)
 Transforming growth factor α (TGF- α)
 Transforming growth factor β (TGF- β)
 Hemopoietin-2, interleukin-3 (IL-3), mast-cell growth factor (MCGF), or erythroid burst-promoting activity (EBPA)
 Interleukin-1 (IL-1)
 Interleukin-2 (IL-2) or T-cell growth factor (TCGF)
 Colony-stimulating factor 1 (CSF-1) or macrophage colony-stimulating factor (MCSF)
 Colony-stimulating factor 2 (CSF-2) or granulocyte-macrophage colony-stimulating factor (GM-CSF)
 Granulocyte colony-stimulating factor (G-CSF)
 Erythropoietin
 Platelet-derived growth factor (PDGF)

Partially characterized growth factors

EGF-like mitogens
 TGF-like growth factors
 PDGF-like growth factors
 Melanocyte growth factor (MGF)
 Hepatopoietin
 Hepatocyte-stimulating factor (HSF)
 Prostate growth factor
 Cartilage-derived growth factor (CDGF)
 Chondrocyte growth factor (CGF)
 Bone-derived growth factor (BDGF)
 Osteosarcoma-derived growth factor (ODGF)
 Neuronal-derived transforming growth factor (ND-TGF)
 Glial growth-promoting factor (GGPF)
 Colostrum basic growth factor (CBGF)
 Endothelial cell growth factor (ECGF)
 Tumor angiogenesis factor (TAF)
 Hemopoietin-1
 IL-3-like proteins
 Eosinophil differentiation factor (EDF) or interleukin-4 (IL-4)
 B-cell growth factor 1 (BCGF) or B-cell stimulating factor 1 (BSF-1)
 B-cell stimulating factor 2 (BSF-2)
 B-cell differentiation factor (BCDF)
 Leukemia-derived growth factor (LDGF)
 Myelomonocytic growth factor (MMGF)
 Macrophage-derived growth factor (MDGF)
 Macrophage-activating factor (MAF)
 Erythroid-potentiating activity (EPA)

Hormones and other growth-regulating agents

Insulin
 Growth hormone (GH) or somatotropin
 Thyroid-stimulating hormone (TSH) or thyrotropin
 Adrenocorticotrophic hormone (ACTH)
 Gonadotropins
 Prolactin
 Melanocyte-stimulating hormone (MSH)
 Placental lactogen (PL)
 Thyroid hormone
 Parathyroid hormone (PTH)
 Calcitonin
 Steroid hormones
 Transferrin
 Interferon (IFN)
 Tumor necrosis factor α (TNF- α)
 Tumor necrosis factor β (TNF- β) or lymphotoxin

(This Table is derived from Pimentel, "Hormones, Growth Factors, and Oncogenes," CRC Press, Boca Raton, pp. 2-3 (1987)).

Suitable formulations include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers.

Accepted pharmaceutical principles are set forth, for example, in A. Osol et al., eds., "Remington's Pharmaceutical Sciences," 16th Ed., Mack Publishing Co., Easton, PA (1980), and descriptions of chemicals, drugs and biologicals which may be employed in accordance with the invention may be found in, for example, "Physicians' Desk Reference," 43d Ed., Medical Economics Co., Inc., Oradell, N.J. (1989); and M. Windholz et al., eds., "The Merck Index," 10th Ed., Merck & Co., Inc., Rahway, N.J. (1983).

By "hormones" is meant generally chemical messengers normally synthesized in the endocrine glands of multicellular organisms and secreted into the extracellular body fluids. Hormones typically are recognized by and bind to specific cellular receptors of their respective target tissues. By "peptide growth factors" or "growth factors" is meant generally hormone-related substances which play an important role in the control mechanisms of growth and development in a diversity of organs and tissues. Unlike classical hormones, growth factors typically are not synthesized in specialized endocrine organs, but are produced and secreted by cells from different tissues in a steady flow to diffuse to responsive cells, frequently located not far from the site of release. Growth factors usually are identified by their ability to induce stimulation of target cell multiplication, and their activity is measured by, for example, assays in which either

the increase in cell populations or the incorporation of labeled thymidine into DNA is determined. A listing of growth factors, hormones, and other growth-regulating agents of exogenous cellular origin which are suitable for use as biologically active substances in accordance with the present invention is provided in Table II. It will be appreciated, of course, that this listing is merely exemplary of suitable agents, and is not intended to be limiting. Further, those of skill will recognize that certain of the listed agents may, in certain circumstances, be unsuitable for use in accordance with the invention.

The clinical therapeutic applications of the biodegradable bioactive membranes of the present invention include, but are not limited to, periodontology, craniofacial defects, orthodontics, ophthalmology and plastic surgery. Generally, the novel materials, compositions and methods of the invention will be useful in virtually any application in which tissue repair or regeneration is desired or desirable.

A preferred clinical therapeutic application of the present invention is in the treatment of wounds, including ulcerations and burn wounds. Effective management and healing such wounds presents a significant challenge to clinicians, in large part because of the risk of complications resulting from infection at the wound site. With respect to full-thickness epithelial burn wounds, for example, the biodegradable bioactive membranes of the present invention may be employed as occlusive wet dressings. Such dressings primarily protect the wound site from bacterial contamination and prevent post-burn contractures, and are often employed during the initial treatment phases while the patient is being readied for skin or skin substitute grafts. When used in this capacity, the biodegradable bioactive membranes of the invention may have a first side, which will be placed in contact with the wound surface, coated with a suitable topical bactericide, bacterio-

stat, or anti-microbial agent, or a combination thereof. This first side may also include other agents selected to enhance migration of cells into the wound site from adjacent intact tissues, as well as suitable growth factors, such as epidermal growth factor, known to be involved in wound repair. The second side of the membrane may be uncoated, or may preferably be coated with a bactericide, bacteriostat, anti-microbial, or other agent. The advantages of the biodegradable bioactive membranes of the invention in this application will include the guidance and enhancement of the rate of migration and proliferation of cells into the wound site and reduced risk of infection. This may allow healing of many wounds without the need for grafting.

When grafting is required, however, the biodegradable bioactive membranes of the present invention will be well suited for use as skin substitutes. The choice of membrane composition and bioactive agents will follow the same general principles as those which apply when the membrane is to be used as a wet occlusive dressing. When employed as a graft, however, the biodegradable bioactive membrane may be sutured into place using appropriate methods known to those of skill. General applicable surgical principles of burn wound treatment and maintenance are set forth, for example, in MacMillanm, *Surgical Clinics N. America* 58(6): (1978); and Parks et al., *Surgical Clinics N. America* 57(5): (1977).

In some applications of the invention, it may be desirable to vary the rate of release of the BAS at different rates from different parts of the membrane. This can be accomplished by overlaying the BAS-coated side of the membrane with a second resorbable membrane layer suited for allowing controlled release of the BAS.

In one embodiment, a square or roughly rectangular-shaped BAS-coated membrane may be visualized as having two opposite edges. The membrane near one edge would have no overlying

second resorbable membrane layer, thus allowing immediate availability of the BAS. The membrane near the second edge would be coated with a plurality of second resorbable membranes. The second resorbable membranes are built up across the BAS-coated membrane in a stepwise fashion.

Thus, for illustrative purposes only, the second edge of the membrane may have 20 layers of second resorbable membranes, whereas the center of the membrane may have less than 20 layers of second resorbable membranes and BAS-coated membrane closest to the first edge of the membrane would have immediately available BAS. This "staggered" layering of second resorbable membranes is shown in Figure 1.

Staggered second resorbable membranes would be applied to one or both sides of the biodegradable bioactive membrane. Application to a side previously coated with, for example, a growth factor, would allow release of the growth factor at different sites at different times, depending on the thickness of the second resorbable membrane layer and the rate of its resorption.

Application of the staggered second resorbable membrane layer to a second side of the biodegradable bioactive membrane, coated with, for example, a bacteriostatic agent, would likewise allow release of the bacteriostatic agent at different sites at different times.

In another embodiment, a roughly circular or rounded BAS-coated membrane may have a region with immediately available BAS, surrounded by a separate region in which the availability of the BAS is delayed by a resorbable membrane layer covering the BAS.

It may be preferable to cover the entire BAS-coated side with a resorbable membrane layer. The controlled release of BAS can then be accomplished by varying the rate of resorption of the resorbable membrane layer.

For example, the layer may contain a gradient of a component which renders the membrane resistant to enzymatic action. Membrane having little or none of this component would permit immediate release of BAS, whereas membrane having a higher concentration of the component would allow delayed release of BAS.

Controlled release of BAS from the membrane may also be accomplished by the incorporation of BAS into the membrane itself. In one embodiment, the membrane comprises two sides. The membrane layer adjacent to one side has incorporated within it one or more BAS. The membrane layer adjacent to the second side has incorporated within it one or more BAS, at least one of which differs from BAS in the membrane layer adjacent to the first side.

For example, the membrane adjacent to the first side may have primary osteogenic factor incorporated within it. The membrane adjacent to the second side may contain a factor to slow epithelial proliferation, such as $TGF\beta$. The membrane would consist of a biodegradable material as described herein, thus allowing controlled release of the BAS as the membrane is degraded.

In another embodiment, controlled release may be accomplished using a membrane comprising two layers. Each layer has incorporated within it one or more BAS, and at least one BAS of one layer differs from the BAS of the second layer. For example, one layer may have primary osteogenic factor incorporated within it. The second layer may contain a factor to slow epithelial growth, such as $TGF\beta$. The membrane layers would consist of biodegradable materials to allow controlled release of the BAS.

The membranes described above may be used to treat a periodontal bony defect. Using the method as described herein, the membrane would be located in the patient's mouth

in such a manner that the osteogenic factor-containing side of the membrane faced and covered the defect.

5 The resorbable membrane as described above may contain other BAS as described herein. Use of such membranes is in accordance with the methods described herein and will depend upon the particular BAS incorporated within the membrane.

The controlled release of BAS can be further accomplished by varying the cross-linking of the polymers in the resorbable membrane.

10 In any of the above embodiments, it may be desirable or advantageous to affix the biodegradable bioactive membrane to the tissue. The means of affixing the membrane will depend upon the composition of the membrane, the site at which the membrane is to be placed, the size of the membrane, and other
15 parameters which will be taken into account by one skilled in the art.

One such method is the use of the biodegradable, bioactive membrane of the invention, having bioadhesive properties. Bioadhesion may be accomplished using, for
20 example, the materials disclosed in U.S. Patent No. Re. 33,093, which is incorporated herein by reference in its entirety.

The membranes of the invention may also be affixed by suturing. Suitable methods of suturing are well-known to one
25 of skill in the art.

Furthermore, methods of affixing may be combined if necessary to achieve a highly secure placement of the membrane.

30 Having described the invention, the manner and method of carrying out the same may be more fully understood by those of skill by reference to the following example, which example is not intended in any manner to limit the scope of the present invention or of the claims directed thereto.

EXAMPLE 1

As a non-limiting example of the use of the present invention, a biodegradable bioactive membrane according to the invention is employed to guide regeneration of human periodontal bony defects. A human subject suffering from interdental bony craters (two-wall lesions) is first given instructions regarding hygiene and prophylaxis, and the size and extent of lesions are noted prior to surgery. An experimental lesion and a comparable lesion which will act as a control are identified. Subgingival curettage at the sites of bony defects is performed fourteen days prior to scheduled surgery. Baseline recordings of gingival recession, probing bone level and probing pocket depth are measured immediately prior to surgery. At the time of surgery, following routine anesthetic procedures, the interdental craters are exposed through the modified Widman technique. Thorough curettage is performed, followed by application of a biodegradable bioactive membrane to the interdental crater at the experimental site. In this example, the biodegradable bioactive membrane is composed of a purified commercially available bovine collagen preparation. The first side of this membrane, which will be placed against the alveolar bone and will cover the defect, is coated with primary osteogenic factor. Primary osteogenic factor is described, for example, in U.S. Patent 4,804,744. The second side of the membrane is coated with erythromycin. The membrane is applied so as to bridge the interdental crater and cover the alveolar bone for a minimum of three millimeters on each side of the defect. Following stabilization of the membrane for about five minutes by gentle finger pressure, the flaps are sutured and dressed with a commercially available periodontal pack. The control defect is treated in an identical fashion, except that the collagen membrane applied is not bioactive. Following surgery, the

evaluation parameters are measured at 12, 24 and 48 weeks. Although no significant differences in gingival recession are observed, there is a noticeable decrease in inflammatory response on the experimental side. Probing bone level and probing pocket depth values, however, reveal a significant improvement in alveolar bone regeneration on the experimental side.

EXAMPLE 2

In this example, a biodegradable bioactive membrane is employed as in Example 1. In this example the first side of this membrane, which will be placed against the alveolar bone and will cover the defect, is coated with primary osteogenic factor. Primary osteogenic factor is described, for example, in U.S. Patent 4,804,744. A second material having anti-microbial properties, such as tetracycline, is also incorporated on this first side. The second side of the membrane is coated with a factor, TGF β , to slow epithelial proliferation. The membrane is applied in the same manner as in Example 1.

EXAMPLE 3

As a second non-limiting example of the use of the present invention, a biodegradable bioactive membrane having at least one side coated with a staggered resorbable membrane according to the invention is employed to guide regeneration of human periodontal bony defects.

A human subject is selected and presurgical procedures carried out according to Example 1. At the time of surgery, following routine anesthetic procedures, the interdental craters are exposed through the modified Widman technique. Thorough curettage is performed, followed by application of a

biodegradable bioactive membrane to the interdental crater at the experimental site.

5 In this example, the biodegradable bioactive membrane is composed of a purified commercially available bovine collagen preparation. The first side of this membrane, which will be placed against the alveolar bone and will cover the defect, is coated with primary osteogenic factor. Some of the primary osteogenic factor is located between the biodegradable bioactive membrane and at least one layer of a second resorbable membrane, in the following manner. At a first edge of the biodegradable bioactive membrane, the primary osteogenic factor remains uncovered. At a second edge of the biodegradable bioactive membrane, the primary osteogenic factor is covered with 20 layers of second resorbable membrane. These layers are built up in a staggered manner between the first and second edge of the biodegradable bioactive membrane.

10 The second side of the biodegradable bioactive membrane is coated with EGF. The membrane is applied so as to bridge the interdental crater and cover the alveolar bone for a minimum of three millimeters on each side of the defect. The first edge of the membrane, having no second resorbable membrane overlay, is placed in proximity to the site of regeneration. The second edge of the membrane, having the maximum thickness of second resorbable membrane overlay, is placed farther from the site of regeneration. Following stabilization of the membrane for about five minutes by gentle finger pressure, the flaps are sutured and dressed with a commercially available periodontal pack.

15 20 25 30 The use of a biodegradable bioactive membrane which is partially covered with a second resorbable membrane allows the gradual release of the primary osteogenic factor as the resorbable membrane is resorbed.

EXAMPLE 4

5 In this example, a biodegradable bioactive membrane is employed as in example 3, except that in this example the second side of the biodegradable bioactive membrane is coated with TGF β .

EXAMPLE 5

10 As a fifth non-limiting example of the use of the present invention, a bioactive, biodegradable membrane is used as an occlusive wet dressing for the treatment of a skin wound.

15 A biodegradable bioactive membrane composed primarily of collagen is prepared and one side of the membrane is coated with epidermal growth factor. The epidermal growth factor layer is at least partially covered with a second resorbable membrane. The epidermal growth factor will be fully exposed and available to the wound sites where immediate regeneration is desired. Delayed or guided regeneration in other areas of the wound sites can be accomplished by controlling release of growth factor to those sites. Release of the growth factor to those sites occurs only after resorption of the resorbable layer protecting the growth factor. Epidermal growth factor is thus immediately available in areas of the wound where immediate regeneration is desired, and sustained or guided regeneration is achieved by delaying the release of growth factor to other sites of the wound.

25 Having now fully described the invention, it will be apparent to one of ordinary skill that many modifications and changes may be made thereto without departing from the spirit and scope thereof, and that such modifications and changes are properly to be considered as falling within the scope of the appended claims.

30

CLAIMSWHAT IS CLAIMED IS:

1. A composition for guided tissue regeneration comprising a biodegradable bioactive membrane comprising a first and a second side, wherein said first side of said membrane comprises one or more biologically active substances not present on said second side.

2. A composition for guided tissue regeneration comprising a biodegradable bioactive membrane comprising two sides, wherein a first and a second side of said membrane each comprises one or more biologically active substances, and wherein at least one of said one or more biologically active substances on said second side is not present on said first side.

3. The composition of claims 1 or 2, wherein said one or more biologically active substances is selected from the group consisting of drugs, hormones, growth factors, peptides and proteins.

4. The composition of claim 3, wherein said peptide or protein is selected from the group consisting of epidermal growth factor, insulin-like growth factor, platelet derived growth factor, fibronectin, laminin, entactin, angiogenesis factor, transforming growth factor- α and bone morphogenic protein.

5. The composition of claims 1 or 2, wherein said first or second side additionally comprises an anti-microbial or other inhibitory agent.

-40-

6. The composition of claims 1 or 2, wherein said biodegradable membrane is comprised of one or more substances selected from the group consisting of collagens, polylactic acid, PELA, laminin, elastin, proteoglycans, nidogen, factor VIII, entactin and fibronectin.

7. A method of guiding regeneration of tissue in animals, including humans, comprising applying in close apposition to said tissue the composition of claims 1-6.

8. The method of claim 7, wherein said tissue is selected from the group consisting of epithelial tissue, bone tissue, ophthalmic tissue, vascular tissue and endothelial tissue.

9. A process for preparing a composition for guided tissue regeneration, comprising conjugating to one or more sides of a biodegradable membrane a biologically active material, to produce a biodegradable bioactive membrane.

10. A composition for guided tissue regeneration comprising a biodegradable bioactive membrane comprising a first and second side, wherein the membrane adjacent to the first side has incorporated within it one or more bioactive substances, the membrane adjacent to the second side has incorporated within it one or more bioactive substances, at least one of which bioactive substances differs from the bioactive substances incorporated in the membrane adjacent to the first side.

11. A method of guiding regeneration of tissue in animals, including humans, comprising applying in close proximity to said tissue the composition of claim 10.

12. A composition for guided tissue regeneration comprising a biodegradable bioactive membrane comprising two layers, wherein each layer has incorporated within it one or more BAS, and at least one BAS of one layer differs from at least one BAS of the second layer.

13. A method of guiding regeneration of tissue in animals, including humans, comprising applying in close proximity to said tissue the composition of claim 12.

14. A composition for guided tissue regeneration comprising a biodegradable bioactive membrane comprising a first and a second side, wherein said first side of said membrane comprises one or more biologically active substances not present on said second side and wherein at least one of said first and second sides is at least partly coated by at least one resorbable membrane layer.

15. The composition of claim 14 wherein at least one of said first and second sides is coated by two or more resorbable membrane layers and wherein said resorbable membrane layers are staggered.

16. A method of guiding regeneration of tissue in animals, including humans, comprising applying in close proximity to said tissue the composition of claim 14.

1/1

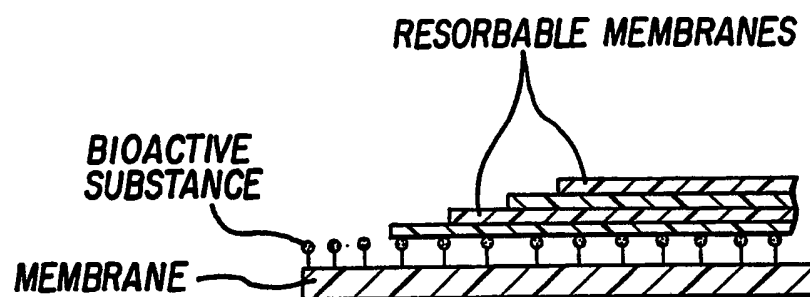


FIG. 1

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/02406

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): A61K 35/32; A61K 37/02; A61K 37/24 US Cl.: 514/12 424/423, 424, 425, 426; 424/445, 447, 448, 449																				
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">US</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">514/12; 424/423, 424, 425, 426; 424/445, 447, 448, 449</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div> <div style="text-align: center; padding: 10px 0;">CAS ONLINE</div>			Classification System	Classification Symbols	US	514/12; 424/423, 424, 425, 426; 424/445, 447, 448, 449														
Classification System	Classification Symbols																			
US	514/12; 424/423, 424, 425, 426; 424/445, 447, 448, 449																			
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category ⁹</th> <th style="border-bottom: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">Y</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">Chemical Abstracts, Volume 105, issued 1986, (Columbus, Ohio, USA), M. Rabaud, Elastin-Based Product and Its Biological Application Particularly As Biomaterial and an Artificial Support, see page 364, column 1, abstract no. 232443m.</td> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">1-8, 10-16</td> </tr> <tr> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">Y</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">SU, A, 353,724 (ARLOZOROV) 09 April 1970 (Note English language abstract SU-483252.U22).</td> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">1, 6, 7, 8, 14, 16</td> </tr> <tr> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">Y</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">US, A, 4,804,744 (SEN) 14 February 1989 (Note abstract and column 5, lines 29-44).</td> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">1-8, 14-16</td> </tr> <tr> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">Y</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">J. Dent. Res., Volume 66 (Special Issue, March) 1987 V. P. Terranova, et al., Reconstituted Basement Membrane Inhibits The Movement Of Gingival Epithelial Cells To Dentin. See page 280, abstract no. 1390.</td> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">1-8, 10-16</td> </tr> <tr> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">Y</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">J. Dent, Res., Volume 65 (Special Issue B) 1986 S. J. Othman, et al., The Effect Of Chlorohexidine (CH) - Containing Coe-pak on wound Healing After Gingivectomy, see page 822, abstract no. 866.</td> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">5, 7, 8</td> </tr> </table>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	Chemical Abstracts, Volume 105, issued 1986, (Columbus, Ohio, USA), M. Rabaud, Elastin-Based Product and Its Biological Application Particularly As Biomaterial and an Artificial Support, see page 364, column 1, abstract no. 232443m.	1-8, 10-16	Y	SU, A, 353,724 (ARLOZOROV) 09 April 1970 (Note English language abstract SU-483252.U22).	1, 6, 7, 8, 14, 16	Y	US, A, 4,804,744 (SEN) 14 February 1989 (Note abstract and column 5, lines 29-44).	1-8, 14-16	Y	J. Dent. Res., Volume 66 (Special Issue, March) 1987 V. P. Terranova, et al., Reconstituted Basement Membrane Inhibits The Movement Of Gingival Epithelial Cells To Dentin. See page 280, abstract no. 1390.	1-8, 10-16	Y	J. Dent, Res., Volume 65 (Special Issue B) 1986 S. J. Othman, et al., The Effect Of Chlorohexidine (CH) - Containing Coe-pak on wound Healing After Gingivectomy, see page 822, abstract no. 866.	5, 7, 8
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³																		
Y	Chemical Abstracts, Volume 105, issued 1986, (Columbus, Ohio, USA), M. Rabaud, Elastin-Based Product and Its Biological Application Particularly As Biomaterial and an Artificial Support, see page 364, column 1, abstract no. 232443m.	1-8, 10-16																		
Y	SU, A, 353,724 (ARLOZOROV) 09 April 1970 (Note English language abstract SU-483252.U22).	1, 6, 7, 8, 14, 16																		
Y	US, A, 4,804,744 (SEN) 14 February 1989 (Note abstract and column 5, lines 29-44).	1-8, 14-16																		
Y	J. Dent. Res., Volume 66 (Special Issue, March) 1987 V. P. Terranova, et al., Reconstituted Basement Membrane Inhibits The Movement Of Gingival Epithelial Cells To Dentin. See page 280, abstract no. 1390.	1-8, 10-16																		
Y	J. Dent, Res., Volume 65 (Special Issue B) 1986 S. J. Othman, et al., The Effect Of Chlorohexidine (CH) - Containing Coe-pak on wound Healing After Gingivectomy, see page 822, abstract no. 866.	5, 7, 8																		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>																				
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">16 July 1990</td> <td style="border-bottom: 1px solid black; padding: 5px; text-align: center;"> <div style="font-size: 1.2em; font-weight: bold;">09 AUG 1990</div> </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">International Searching Authority</td> <td style="border-bottom: 1px solid black; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="padding: 5px;">ISA/US</td> <td style="padding: 5px; text-align: center;"> Fatemeh Moezi </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	16 July 1990	<div style="font-size: 1.2em; font-weight: bold;">09 AUG 1990</div>	International Searching Authority	Signature of Authorized Officer	ISA/US	 Fatemeh Moezi										
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report																			
16 July 1990	<div style="font-size: 1.2em; font-weight: bold;">09 AUG 1990</div>																			
International Searching Authority	Signature of Authorized Officer																			
ISA/US	 Fatemeh Moezi																			

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	<p>The Journal of Biological Chemistry, Volume 263, no. 15, Issed 25 May 1988, M. Jones, Influence Of The Subendothelial Basement Membrane Components On Fibrin Assembly, pages 7043-7048.</p>	1-16
---	--	------

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . . . , because they relate to subject matter ¹² not required to be searched by this Authority, namely:
2. ☐ Claim numbers . . . , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:
3. ☐ Claim numbers . . . , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.